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Group V Phospholipase A_2 in Bone Marrow-derived Myeloid Cells and Bronchial Epithelial Cells Promotes Bacterial Clearance after *Escherichia coli* Pneumonia*[S]

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Background: GV $sPLA_2$ hydrolyzes bacterial phospholipids. Myeloid and non-myeloid cells in lung express GV $sPLA_2$.
Result: Deletion of GV $sPLA_2$ impairs leukocyte accumulation and bacterial clearance after lung infection with *E. coli*.
Conclusion: GV $sPLA_2$ regulates the innate immune response to *E. coli* pneumonia.
Significance: Inhibiting GV $sPLA_2$ to treat inflammatory diseases could impair the immune response to bacterial infection.

Group V-secreted phospholipase A_2 (GV $sPLA_2$) hydrolyzes bacterial phospholipids and initiates eicosanoid biosynthesis. Here, we elucidate the role of GV $sPLA_2$ in the pathophysiology of *Escherichia coli* pneumonia. Inflammatory cells and bronchial epithelial cells both express GV $sPLA_2$ after pulmonary *E. coli* infection. $GV^{-/-}$ mice accumulate fewer polymorphonuclear leukocytes in alveoli, have higher levels of *E. coli* in bronchoalveolar lavage fluid and lung, and develop respiratory acidosis, more severe hypothermia, and higher IL-6, IL-10, and TNF-α levels than $GV^{+/+}$ mice after pulmonary *E. coli* infection. Eicosanoid levels in bronchoalveolar lavage are similar in $GV^{+/+}$ and $GV^{-/-}$ mice after lung *E. coli* infection. In contrast, $GV^{+/+}$ mice have higher levels of prostaglandin D_2 (PGD_2), $PGF_{2\alpha}$, and 15-keto-PGE_2 in lung and express higher levels of ICAM-1 and PECAM-1 on pulmonary endothelial cells than $GV^{-/-}$ mice after lung infection with *E. coli*. Selective deletion of GV $sPLA_2$ in non-myeloid cells impairs leukocyte accumulation after pulmonary *E. coli* infection, and lack of GV $sPLA_2$ in either bone marrow-derived myeloid cells or non-myeloid cells attenuates *E. coli* clearance from the alveolar space and the lung parenchyma. These observations show that GV $sPLA_2$ in bone marrow-derived myeloid cells as well as non-myeloid cells, which are likely bronchial epithelial cells, participate in the regulation of the innate immune response to pulmonary infection with *E. coli*.

Bacterial pneumonia is a leading cause of morbidity and mortality (1, 2) Host-defenses against bacterial pneumonia include anatomic barriers, mucociliary clearance, anti-microbial secretions, and an innate immune response that is characterized by the mobilization of leukocytes, including alveolar and circulating macrophages, monocytes, and polymorphonuclear leukocytes (PMN)[5] to the alveolar space (3). Activated leukocytes may release their granule contents, including secreted phospholipase A_2 ($sPLA_2$) enzymes, in response to bacterial infec-

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This paper is dedicated to the memory of Helmut Schmidt.
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[5] The abbreviations used are: PMN, polymorphonuclear leukocyte; $sPLA_2$, secreted phospholipase A_2; cfu, colony-forming unit; ICAM, intercellular adhesion molecule; GV, Group V; PG, prostaglandin; BAL, bronchoalveolar lavage; VCAM, vascular cell adhesion molecule; ANOVA, analysis of variance; HETE, hydroxyeicosatetraenoic acid; MCP-1, monocyte chemoattractant protein-1; LTB_4, leukotriene B_4; dh, di-hydro; Tx, thromboxane; PECAM, platelet endothelial cell adhesion molecule.

tion (4). To date, 10 enzymatically active mammalian sPLA$_2$ enzymes have been identified, namely Group IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XIIA sPLA$_2$ (5). Of these enzymes, Groups V and X sPLA$_2$ are components of the granules of human PMN, and most of the PLA$_2$ activity in the extracellular fluid of PMN stimulated with the bacterial tripeptide formylmethionylleucylphenylalanine is attributable to GV sPLA$_2$ (6). The observations that GV sPLA$_2$ can hydrolyze Gram-negative bacterial phospholipids (6) and that phagocytosis by peritoneal macrophages is modulated by GV sPLA$_2$ (7) suggests that GV sPLA$_2$ participates in the killing of bacteria by leukocytes (5). GV sPLA$_2$ can be expressed by macrophages (7, 8), mast cells (9, 10), airway smooth muscle cells (11), and bronchial epithelial cells (12) in mice and by alveolar macrophages, bronchial epithelial cells, and interstitial fibroblasts in patients with pneumonia, where expression of GV sPLA$_2$ correlates with the severity of inflammation (13). Overexpression of GV sPLA$_2$ leads to increased PGE$_2$ biosynthesis by cultured bronchial epithelial cells (13) and to surfactant degradation, respiratory distress, and neonatal lethality in transgenic mice that constitutively express the enzyme under an actin promotor (14). Together, these observations suggest a central role for GV sPLA$_2$ in the innate immune response to bacterial pneumonia.

sPLA$_2$ enzymes, including GV sPLA$_2$, mediate biological responses in part through the release of free arachidonic acid and its biologically active metabolites, including prostaglandins, leukotrienes, and thromboxanes (15). The functional importance of GV sPLA$_2$ in eicosanoid biosynthesis is established by the observations that LTB$_4$ and PGE$_2$ production is ~50% lower in macrophages from mice with targeted deletion of GV sPLA$_2$ (GV$^{-/-}$ mice) than from GV$^{+/+}$ mice (16) and that BAL fluid from mice that overexpress GV sPLA$_2$ contains about 2-fold more PGE$_2$ than wild type mice (14). sPLA$_2$ enzymes also regulate biological responses by generating 1-*O*-alkyl-lysophosphatylcholine, the precursor of platelet activating factor (17), and through binding to specific mannose-type sPLA$_2$ receptors (18, 19).

The purpose of this study was to identify the role of GV sPLA$_2$ in the innate immune response to *Escherichia coli* pneumonia, because pulmonary infection with *E. coli* causes significant morbidity and mortality, especially in very young, elderly, and immunocompromised individuals (1, 20). Our results demonstrate that GV sPLA$_2$ in bone marrow-derived myeloid cells and in non-myeloid cells that are likely bronchial epithelial cells participates in the regulation of bacterial clearance after lung infection with *E. coli*.

EXPERIMENTAL PROCEDURES

Reagents were from Sigma unless otherwise stated. Construction of the GV sPLA$_2$-deficient mouse line, backcrossed to the C57BL/6 background for 10 generations, was carried out as described (16). Heterozygous breeding pairs (GV$^{+/-}$) were used to generate GV$^{-/-}$ and GV$^{+/+}$ littermates. Housing and experimental procedures were approved by the Animal Care Committee of the University Health Network and were in accordance with the Guide for the Care and Use of Laboratory Animals Research Statutes, Ontario (1980).

E. coli Pneumonia Model—Intra-tracheal administration of live *E. coli* (strain DH5α, 1×10^9 colony-forming units (cfu) in 50 μl of PBS) was performed as described by our group (22). After 3, 18, or 48 h, mice were re-anesthetized, heparinized arterial blood was collected by abdominal aortic puncture (for arterial blood gas analysis), and mice were euthanized by transection of the abdominal aorta. BAL fluid was aspirated after infusion of the trachea with 1 ml of ice-cold PBS (6 times) and centrifuged, and the cell-free supernatant was stored at −80 °C until analysis. After resuspension in PBS, leukocytes counts in BAL were determined. Bacterial viability in BAL fluid, lung, spleen, kidney, liver, and blood were assessed by serial dilution culture, as described (22). All samples were analyzed in triplicate.

Bone Marrow Transplantation and Generation of Chimeric Mice—Bone marrow was isolated from male GV$^{+/+}$ or GV$^{-/-}$ mice killed by spinal cord displacement. Female recipient GV$^{+/+}$ mice were irradiated with 2 sub-lethal doses of 9.5 gray (Gammacell 40 ^{137}Cs γ-irradiation source), which induces agranulocytosis (23), injected with 8×10^6 bone marrow cells from donor male mice (via the tail vein), and kept in microisolator cages for 10 weeks to allow full humoral reconstitution (24). The efficiency of bone marrow transplantation was estimated by measuring the ratio of SYR (a gene located on the Y chromosome) to GAPDH DNA by real time PCR and was consistently >95%. The mouse SYR primers 5′-GTACAACCTTCTGCAGTGGGACAGG-3′ and 5′-GCTGGTTTTTGGAGTACAGGTGTGC-3′ were used.

LC/MS/MS Analysis of Eicosanoids in BAL Fluid and in Lungs after BAL Fluid Harvest from GV$^{+/+}$ and GV$^{-/-}$ Mice—After the addition of deuterated PGE$_2$, PGD$_2$, PGF$_{2\alpha}$, TxB$_2$ and 6-keto-PGF$_{1\alpha}$ (Cayman Chemical Co., Ann Harbor, MI), BAL fluid samples and lung (after BAL fluid harvest) were subjected to liquid-liquid extraction (25). For LC/MS/MS analysis, samples were separated on a Synergi Hydro-RP column and precolumn (Phenomenex, Aschaffenburg, Germany) as described by our group (25). The API 4000 triple quadrupole tandem mass spectrometer was operated in negative ion mode, and multiple reaction monitoring was used for quantification with Analyst Software Version 1.4 (Applied Biosystems, Darmstadt, Germany).

ELISA for Selected Prostaglandins in BAL Fluid from GV$^{+/+}$ and GV$^{-/-}$ Mice—Levels of cysteinyl leukotrienes, PGD$_2$ methoxylamine hydrochloride (PGD$_2$-MOX), PGE$_2$, PGE$_2$ metabolites, and LTB$_4$ present in BAL fluid collected from the lungs of GV$^{+/+}$ or GV$^{-/-}$ mice were independently measured using specific ELISA kits (Cayman Chemical) according to the manufacturer's instructions.

Histology, Immunohistochemistry, Immunoblotting, and PCR—Lungs were inflated, placed in optimum cutting temperature-embedding compound (Sakura Finetek USA, Torrance, CA), cryo-sectioned, and acetone-fixed for immunohistologic analysis. Rabbit anti-murine GV sPLA$_2$, ICAM-1, ICAM-2, PECAM-1, VCAM, and E-selectin (Santa Cruz Biotechnology, Santa Cruz, CA) were used to assess the tissue distribution of these proteins (25). Immunoblots and PCR for GV sPLA$_2$ were carried out as described by our group (6).

Flow Cytometry—CD11b and CD18 surface expression on PMNs isolated from the BAL fluid of GV$^{+/+}$ or GV$^{-/-}$ mice was measured by FACS analysis using fluorescence-conjugated anti-CD11b-FITC or CD18-PE (BD Biosciences) antisera, respectively. To analyze PMN apoptosis, cells were stained with annexin V and propidium iodide (ApoAlert AnnexinV-FITC Apoptosis kit, Clontech Laboratories, Mountain View, CA) based on the manufacturer's protocol and analyzed by FACS (FACScan flow cytometer).

Measurement of Cytokine and Chemokine Levels—Levels of TNF-α, INF-γ, IL-1β, IL-6, IL-10, IL-12p70, and MCP-1 levels in plasma were assessed using cytometric bead array kits (BD Biosciences), as described (6).

Measurement of Bactericidal Activity of Recombinant Mouse GV on Live E. coli—The bactericidal effects of recombinant mouse GV sPLA$_2$ (35 μg/ml) were tested against *E. coli* (strain DH5α, 1×10^7 cfu in 50 μl of reaction) exactly as described previously (7). The solutions were incubated with bacteria at 37 °C for 120 min with or without 0.5% normal serum, plated on brain heart infusion agar, and grown for 24 h to measure cfu. The bactericidal tests are given as mean values in cfu of five independent experiments for each group.

Isolation of Alveolar Macrophages—Resident alveolar macrophages from GV$^{+/+}$ or GV$^{-/-}$ mice were isolated from BAL fluid by centrifugation, resuspended in culture medium A (DMEM, 1% penicillin-streptomycin, 1% L-glutamine, 10% FCS, 0.1% Fungizone, and 5 mM EDTA), allowed to adhere to tissue culture plates (1 h), and washed twice. Cells were then treated for 18 h with vehicle or LPS (10 μg/ml) or for 3, 6, or 18 h with vehicle or live *E. coli* (strain DH5α, 1×10^8 cfu/ml) in medium A without antibiotics.

PLA$_2$ Assay—Extracellular PLA$_2$ activity was determined by measuring the amounts of free [1-^{14}C]oleic acid released from [1-^{14}C]oleic acid-labeled *E. coli* according to the protocol developed by Elsbach and Weiss (26) The reaction was carried out in a total volume of 1.5 ml of 0.1 M Tris buffer, pH 7.5, containing 7 mM $CaCl_2$, 10 mg of fatty acid-free bovine serum albumin, and 2.8×10^8 radiolabeled *E. coli* (corresponding to 5.6 nmol of phospholipid). After a 30-min incubation at 37 °C, the reaction was terminated by filtration through a 0.45-μm Millipore filter, and the released [1-^{14}C]oleic acid bound to the bovine serum albumin carrier was estimated by liquid scintillation counting, as described (27). All assays were performed in the excess of substrate and corrected for non-enzymatic hydrolysis. 1 unit of PLA$_2$ activity was defined as the amount of enzyme that hydrolyzed 56 pmol of phospholipid substrate in 30 min at 37 °C, which corresponds to 1% of the total *E. coli* substrate.

Statistical Analysis—Statistical analysis was performed by analysis of variance (ANOVA) or 2-way ANOVA to evaluate the effect of group, time, and group-time interactions as appropriate. Post hoc analysis by unpaired Student's *t* test, two-tailed, unequal variance was only done if the results of the ANOVA or two-way ANOVA (group, time, or group-time interaction) were significant. A value of $p < 0.05$ was accepted as statistically significant. When multiple comparisons were made, a Bonferroni correction was applied. In total, 65 GV$^{+/+}$ mice, 68 GV$^{-/-}$ mice, 15 GV$^{+/+}$ mice with GV$^{+/+}$ bone marrow, 15 GV$^{+/+}$ mice with GV$^{-/-}$ bone marrow, 18 GV$^{-/-}$ mice with GV$^{+/+}$ bone marrow, and 19 GV$^{-/-}$ mice with GV$^{-/-}$ bone marrow were used in this study.

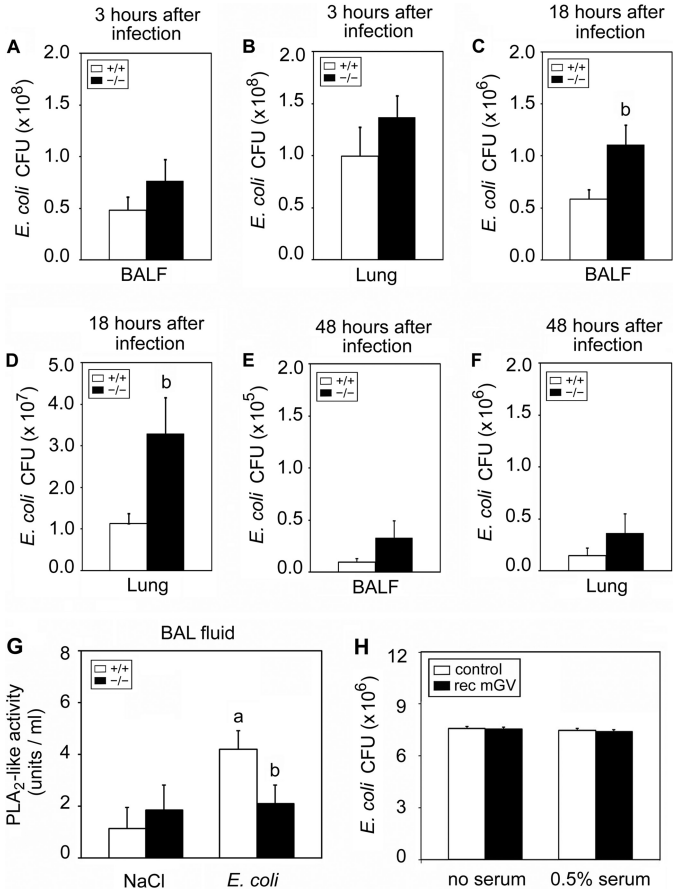

FIGURE 1. **Impaired bacterial clearance in GV$^{-/-}$ mice after pulmonary *E. coli* infection.** Shown are bacterial levels in BAL fluid 3 h (*BALF*, *A*), 18 h (*C*), and 48 h (*E*) after injection of 10^9 cfu of *E. coli* into the trachea of GV$^{+/+}$ (*open bars*) and GV$^{-/-}$ (*filled bars*) mice and in lung (after BAL fluid harvest) 3 h (*B*), 18 h (*D*), and 48 h (*F*) after the same injection. PLA$_2$ activity in BAL fluid (*G*) and bacterial killing after co-incubation of live *E. coli* with recombinant mouse GV sPLA$_2$ *in vitro* (*H*) are shown. The mean specific PLA$_2$ activity for recombinant mouse GV (*mGV*) sPLA$_2$ was measured at 3.2 ± 0.5 units/ng. *a*, $p < 0.05$, *E. coli versus* NaCl; *b*, $p < 0.05$, GV$^{+/+}$ *versus* GV$^{-/-}$, ANOVA followed by paired *t* tests, 2-tailed, unequal variance, serial dilution culture, $n \geq 20$ per group.

RESULTS

Impaired Pulmonary Bacterial Clearance in GV$^{-/-}$ Mice after Lung Infection with E. coli—To assess the role of GV sPLA$_2$ in the innate immune response to pulmonary bacterial infection, we injected 1×10^9 live *E. coli* into the trachea of GV$^{+/+}$ and GV$^{-/-}$ mice. Three hours after pulmonary *E. coli* infection, bacterial levels were similar in BAL fluid (Fig. 1*A*) and in lung tissue after BAL fluid harvest in GV$^{+/+}$ and GV$^{-/-}$ mice (Fig. 1*B*). In contrast, 18 h after pulmonary *E. coli* infection, bacterial levels were ~2-fold higher in BAL fluid (Fig. 1*C*) and 3-fold higher in lung (after aspiration of BAL fluid) in GV$^{-/-}$ than GV$^{+/+}$ mice (Fig. 1*D*). There was no significant difference in bacterial levels in arterial blood, liver, kidney, or spleen between GV$^{+/+}$ and GV$^{-/-}$ mice 18 h after pulmonary *E. coli* infection (supplemental Fig. 1). By 48 h after infection, levels of *E. coli* in BAL fluid (Fig. 1*E*) and lungs (Fig. 1*F*) had

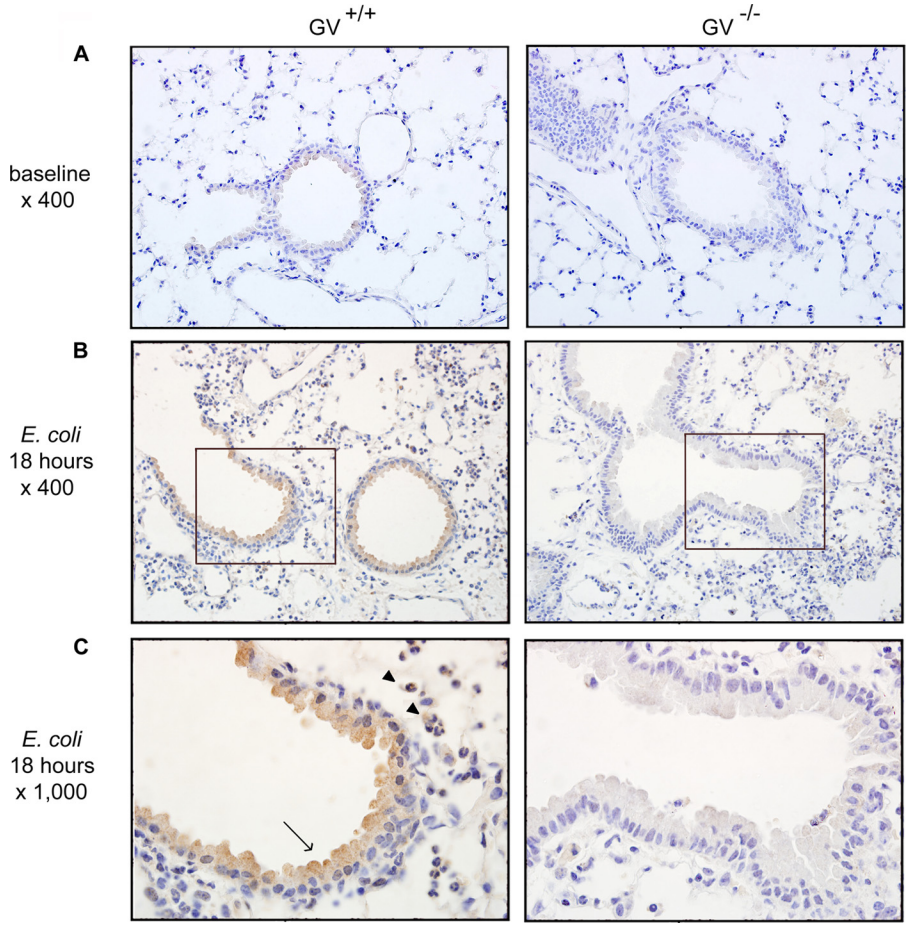

FIGURE 2. **GV sPLA$_2$ is expressed in inflammatory cells and in bronchial epithelial cells after pulmonary *E. coli* infection.** Immunohistochemical analysis (×400) with rabbit anti-mouse GV sPLA$_2$ antiserum before (*A*) and 18 h after intra-tracheal injection of 10^9 cfu of *E. coli* (*B*) into GV$^{+/+}$ and GV$^{-/-}$ mice. *C*, shown is a magnified view (×1000) of the *boxed-in* areas shown in *panel B*. Short *arrowheads* identify leukocytes, and *long arrowheads* identify bronchiolar epithelial cells that stained positively with the anti-mouse GV sPLA$_2$ antiserum after *E. coli* inoculation. Results are representative of $n \geq 8$ independent experiments for each group.

decreased significantly and were not statistically different between GV$^{+/+}$ and GV$^{-/-}$ mice.

PLA$_2$ activity in the BAL fluid was low in GV$^{+/+}$ and GV$^{-/-}$ mice before infection. Eighteen hours after infection with *E. coli*, PLA$_2$ activity in BAL fluid increased significantly in GV$^{+/+}$ mice and was significantly higher in GV$^{+/+}$ than GV$^{-/-}$ mice (Fig. 1*G*). Conversely, co-incubation of catalytically active recombinant mouse GV sPLA$_2$ with live *E. coli in vitro* had no effect on bacterial viability (Fig. 1*H*), a finding consistent with previous results (28).

GV sPLA$_2$ protein was not identified in the lungs of GV$^{+/+}$ or GV$^{-/-}$ mice before infection with *E. coli* (Fig. 2*A*). Eighteen hours after pulmonary *E. coli* infection, GV sPLA$_2$ protein was identified in bronchial epithelial cells and inflammatory cells in GV$^{+/+}$ mice (Fig. 2, *B* and *C*). GV sPLA$_2$ protein was not identified in GV$^{-/-}$ mice after intra-tracheal administration of *E. coli* (Fig. 2, *B* and *C*). GV$^{+/+}$ and GV$^{-/-}$ mice both developed a marked inflammatory response in the alveolar space after infection with *E. coli*, but there was no difference in pulmonary architecture between these mice (supplemental Fig. 2). Taken together, these results show that pulmonary infection with *E. coli* stimulates the expression of GV sPLA$_2$ protein in both bronchial epithelial cells and inflammatory cells in the lung and that targeted deletion of GV sPLA$_2$ attenuates PLA$_2$ activity in BAL fluid and impairs pulmonary *E. coli* clearance *in vivo*.

GV$^{-/-}$ Mice Develop Respiratory Acidosis and Systemic Inflammation after Pulmonary E. coli Infection—Pulmonary infection with *E. coli* led to a decrease in pH (Fig. 3*A*) and an increase in pCO_2 (Fig. 3*B*) in the arterial blood of GV$^{-/-}$ in comparison with GV$^{+/+}$ mice. No differences in HCO_3 levels in arterial blood were observed between GV$^{+/+}$ and GV$^{-/-}$ mice (not shown), and GV$^{+/+}$ and GV$^{-/-}$ mice developed similar levels of arterial hypoxemia after *E. coli* infection (Fig. 3*C*). Sustained hypothermia, a characteristic physiologic response of mice to bacterial infection (29), was observed in both GV$^{+/+}$ and GV$^{-/-}$ mice after intra-tracheal injection of *E. coli* but was more pronounced in GV$^{-/-}$ mice, whereas transient and mild hypothermia was observed in GV$^{+/+}$ and GV$^{-/-}$ mice after intra-tracheal injection of NaCl (Fig. 3*D*). GV$^{-/-}$ mice also exhibited less mobility and grooming than GV$^{+/+}$ mice after pulmonary *E. coli* infection than GV$^{+/+}$ mice (not shown).

Sustained hypothermia in GV$^{-/-}$ mice was associated with higher levels of IL-6, IL-10, and TNF-α in arterial blood in comparison with GV$^{+/+}$ mice after pulmonary *E. coli* infection (Fig. 3, *E–G*). In contrast, pulmonary *E. coli* infection increased MCP-1 levels in arterial blood in GV$^{+/+}$ and GV$^{-/-}$ mice to a

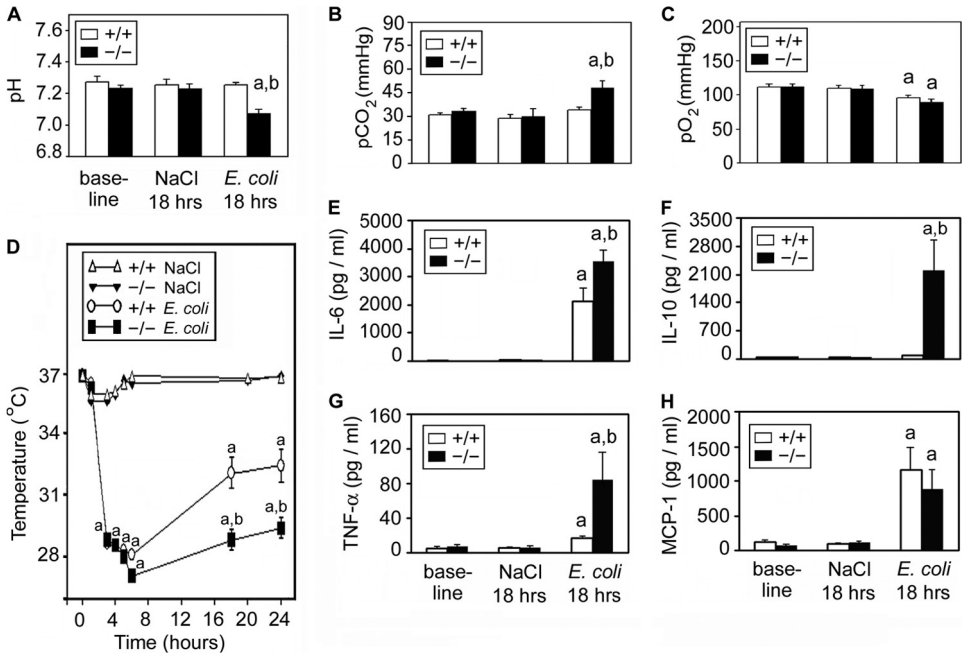

FIGURE 3. **Respiratory acidosis, hypothermia, and systemic inflammation in GV$^{-/-}$ mice after intra-tracheal *E. coli* injection.** Shown is pH (*A*), pCO$_2$ (mm Hg) (*B*), and pO$_2$ (mmHg) (*C*) of arterial blood 18 h after injection of 10^9 cfu of *E. coli* into the trachea of GV$^{+/+}$ (*open bars*) and GV$^{-/-}$ (*filled bars*) mice. *D*, shown is body temperature of GV$^{+/+}$ (*open triangle*) and GV$^{-/-}$ (*filled triangle*) mice after intra-tracheal injection of NaCl and of GV$^{+/+}$ (*open oval*) and GV$^{-/-}$ (*filled rectangle*) mice after intra-tracheal injection of 10^9 cfu *E. coli*. Shown are IL-6 (*E*), IL-10 (*F*), TNF-α (*G*), and MCP-1 (*H*) levels in arterial blood, pg/ml, at base line and 18 h after intra-tracheal injection of NaCl or 10^9 cfu of *E. coli* into GV$^{+/+}$ (*open bars*) and GV$^{-/-}$ (*filled bars*) mice. *a*, $p < 0.05$, *E. coli versus* sham operation or base line; *b*, $p < 0.05$, GV$^{+/+}$ *versus* GV$^{-/-}$, ANOVA followed by paired *t* test, 2-tailed, unequal variance, $n \geq 8$ independent experiments for each group.

TABLE 1

Complete blood counts and differential of arterial blood after intra-tracheal injection of NaCl or *E. coli*

$n \geq 8$ independent experiments for *E. coli*, and ≥ 3 independent experiments for NaCl-injected mice.

	GV$^{+/+}$ NaCl	GV$^{-/-}$ NaCl	GV$^{+/+}$ *E. coli*	GV$^{-/-}$ *E. coli*
Hemoglobin (g/liter)	123 ± 12	126 ± 14	114 ± 4.0	121 ± 4.0
White blood cells (10^8/liter)	4.1 ± 0.1	6.0 ± 0.2	5 ± 1.3	9.8 ± 3.5[a]
Platelets (10^8/liter)	2.8 ± 0.2	1.9 ± 0.2	6.1 ± 2.8	3.5 ± 2.1
PMN (10^7/liter)	4.5 ± 0.05	18.5 ± 2.3[a]	15.0 ± 2.5	11.7 ± 2.5
Lymphocytes (10^7/liter)	34.1 ± 1.5	38.5 ± 2.5	29.5 ± 3.0	83.0 ± 2.0[a,b]
Monocytes (10^7/liter)	1.2 ± 0.1	1.2 ± 0.1	0.75 ± 0.15	0.90 ± 0.3
Eosinophils (10^7/liter)	1.3 ± 0.1	0.3 ± 0.1	0.0 ± 0.25	0.7 ± 0.4

[a] $p < 0.05$, GV$^{+/+}$ *vs.* GV$^{-/-}$ mice, NaCl, or *E. coli*.
[b] $p < 0.05$ NaCl *vs. E. coli*.

similar degree (Fig. 3*H*) and had no effect on arterial IL-12 or INF-γ levels (not shown). GV$^{-/-}$ mice had higher levels of circulating white blood cells, predominantly due to an increase in circulating lymphocyte counts in these mice (Table 1). Hypothermia and increased IL-6, IL-10, and TNF-α levels in arterial blood are consistent with the development of a systemic inflammatory response in GV$^{-/-}$ mice after lung infection with *E. coli*.

Decreased Leukocyte Accumulation in BAL Fluid and Decreased Lung ICAM-1 and PECAM-1 Expression in GV$^{-/-}$ Mice after Pulmonary E. coli Infection—Three hours after intra-tracheal injection of NaCl or *E. coli*, cell counts in BAL fluid were similar in GV$^{+/+}$ and GV$^{-/-}$ mice (Fig. 4*A*), and the cells in BAL fluid were virtually all macrophages (Fig. 4, *B* and *C*). Conversely, 18 h after pulmonary *E. coli* infection, cell counts in BAL fluid were significantly lower in GV$^{-/-}$ than GV$^{+/+}$ mice (Fig. 4*D*), and the cells in BAL fluid were predominantly PMN (Fig. 4, *E* and *F*). By 48 h after intra-tracheal injection of *E. coli*, cell counts in BAL fluid remained elevated in comparison with NaCl-treated mice but were not statistically different between GV$^{+/+}$ and GV$^{-/-}$ mice (Fig. 4*G*), and PMN continued to be the dominant cells in BAL fluid in these mice (Fig. 4, *H* and *I*). Impaired accumulation of PMN in the lungs of GV$^{-/-}$ mice does not appear to be due to impaired expression of activated surface CD11b/CD18 on PMN (Fig. 4*J*), which is necessary for PMNs to migrate from blood into the alveolar space after lung infection with *E. coli* (30) or to accelerated apoptosis of PMN from GV$^{-/-}$ in comparison with GV$^{+/+}$ mice (Fig. 4*K*). In separate experiments, we confirmed that murine PMNs express GV sPLA$_2$ mRNA and protein and showed that exposure to LPS or to live *E. coli in vitro* induces GV sPLA$_2$ mRNA expression by murine alveolar macrophages harvested from GV$^{+/+}$ mice (supplemental Fig. 3, *A–D*).

Next, we assessed the expression of a panel of adhesion molecules that participate in leukocyte transmigration from blood to the alveolar space by immunoblotting (Fig. 5, *A–D*) and immunohistochemistry (Fig. 5, *E–J*). E-selectin levels in lung increased in both GV$^{+/+}$ and GV$^{-/-}$ mice after infection with *E. coli*, but there was no difference in lung E-selectin levels between these mice (Fig. 5*B*). Conversely, expression of

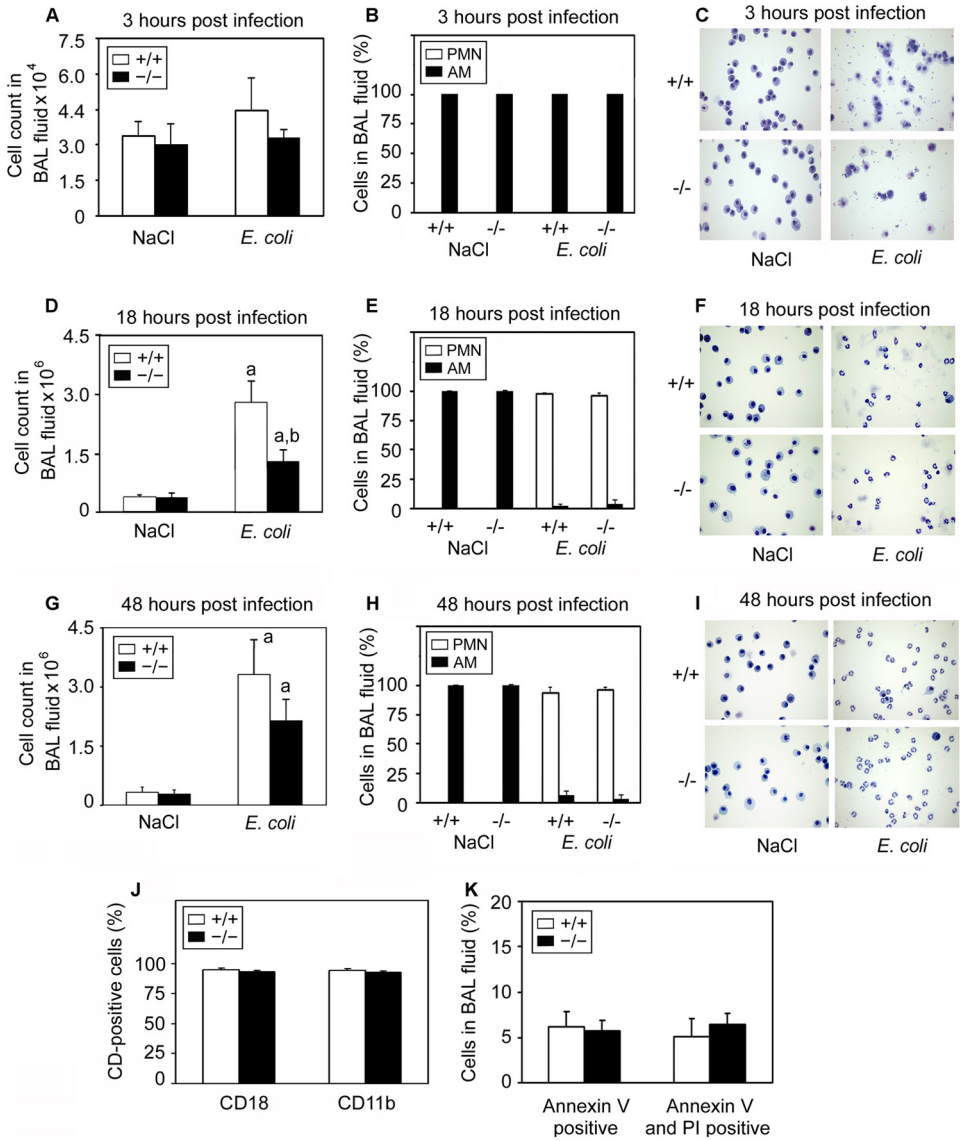

FIGURE 4. **Decreased leukocyte accumulation in BAL fluid in GV$^{-/-}$ mice after lung infection with *E. coli*.** Shown is cell count in BAL fluid of GV$^{+/+}$ (*open bars*) and GV$^{-/-}$ (*filled bars*) mice (*A*), percent of BAL fluid cells that is PMN (*open bars*) or alveolar macrophages (*AM*; *closed bars*) (*B*), and cytospin of BAL fluid 3 h after intra-tracheal injection of 0.9% NaCl or 10^9 cfu of *E. coli* (*C*). Shown is cell count in BAL fluid of GV$^{+/+}$ (*open bars*) and GV$^{-/-}$ (*filled bars*) mice (*D*), percent of BAL fluid cells that is PMN (*open bars*) or alveolar macrophages (*closed bars*) (*E*), and cytospin of BAL fluid 18 h after intra-tracheal injection of 0.9% NaCl or 10^9 cfu of *E. coli* (*F*). Shown is cell count in BAL fluid of GV$^{+/+}$ (*open bars*) and GV$^{-/-}$ (*filled bars*) mice (*G*), percent of BAL fluid cells that is PMN (*open bars*) or alveolar macrophages (*closed bars*) (*H*), and cytospin of BAL fluid 48 h after intra-tracheal injection of 0.9% NaCl or 10^9 cfu of *E. coli*, $n \geq 20$ per group (*I*). Shown is surface expression of activated CD11b and CD18 (*J*) and annexin V expression on the surface of cells in BAL fluid from GV$^{+/+}$ and GV$^{-/-}$ mice harvested 18 h after *E. coli* infection (*K*). *Open bars*, GV$^{+/+}$; *filled bars*. GV$^{-/-}$. *PI*, propidium iodide. $n \geq 6$ per group. *a*, $p < 0.05$, *E. coli versus* NaCl; *b*, $p < 0.05$, GV$^{+/+}$ *versus* GV$^{-/-}$, ANOVA followed by paired *t* tests, 2 tailed, unequal variance, $n \geq 8$ independent experiments for each group.

ICAM-1 (Fig. 5*C*) and PECAM-1 (Fig. 5*D*) in lung (after BAL fluid harvest) increased significantly in GV$^{+/+}$ but not in GV$^{-/-}$ mice after infection with *E. coli*.

Immunohistochemical analysis with an irrelevant isotype control antibody did not result in staining of lung from GV$^{+/+}$ or GV$^{-/-}$ mice (Fig. 5, *E* and *F*) and confirmed higher expression of ICAM-1 (Fig. 5, *G* and *I*) and PECAM-1 (Fig. 5, *H* and *J*) in the lungs of GV$^{+/+}$ than GV$^{-/-}$ mice 18 h after lung infection with *E. coli*. Pulmonary *E. coli* infection had no effect on lung VCAM-1 or ICAM-2 levels (densitometry analysis not shown). Taken together, these results show that deletion of GV sPLA$_2$ impairs ICAM-1 and PECAM-1 expression in the lung and attenuates PMN accumulation in alveoli after pulmonary *E. coli* infection.

Altered Eicosanoid Production in the Lung Parenchyma of GV$^{-/-}$ Mice after E. coli Infection—As eicosanoids can stimulate the recruitment of inflammatory cells into the lungs, we assessed the levels of a panel of eicosanoids in BAL fluid and in the lungs after BAL fluid harvest from GV$^{+/+}$ and GV$^{-/-}$ mice after intra-tracheal injection of NaCl or *E. coli*. Infection with *E. coli* stimulated an increase in the levels of PGD_2, PGE_2, TxB_2 (a breakdown product of TxA_2), $PGF_{2\alpha}$, 15-keto PGE_2, 15-keto $PGF_{2\alpha}$, 13,14-dh-15-keto PGE_2, 13,14-dh-15-keto PGA_2, LTB_4, and 5-, 11-, and 12-HETE in BAL fluid (Fig. 6, *A–L*), as measured by LC-MS/MS, in comparison with sham operation in both GV$^{+/+}$ and GV$^{-/-}$ mice. No difference in the levels of any eicosanoids in BAL fluid were

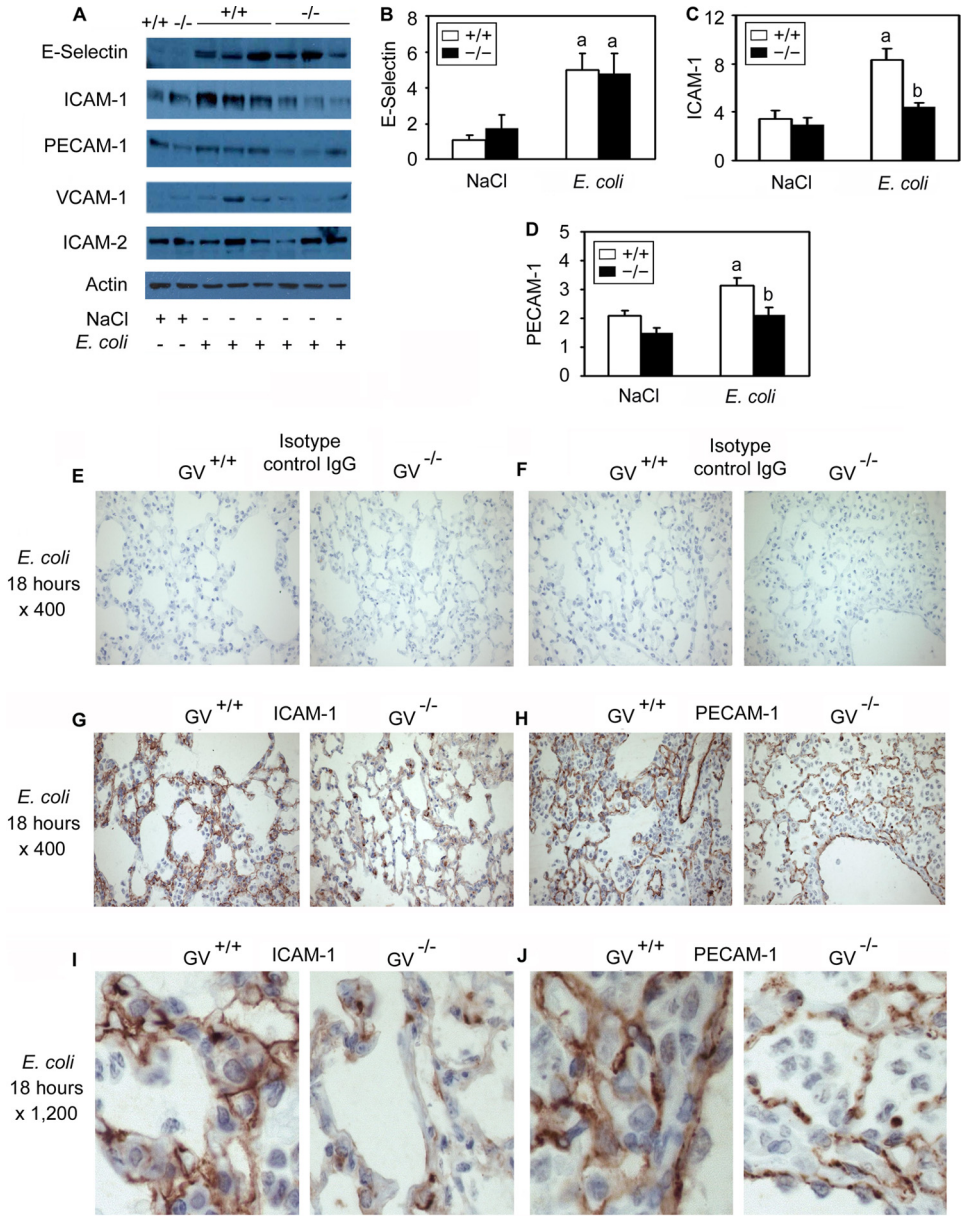

FIGURE 5. **Lack of GV sPLA$_2$ attenuates ICAM-1 and PECAM-1 expression in lung after *E. coli* infection.** *A*, shown is an immunoblot analysis of selected adhesion molecules in lung tissue after intra-tracheal injection of 0.9% NaCl or 10^9 cfu of *E. coli*. Representative lung tissue samples from one GV$^{+/+}$ and one GV$^{-/-}$ mouse after NaCl and from three GV$^{+/+}$ and three GV$^{-/-}$ mice after *E. coli* infection are shown. Densitometric analysis of E-Selectin (*B*), ICAM-1 (*C*), and PECAM-1 (*D*) levels in lung tissue, normalized to actin levels, in GV$^{+/+}$ (*open bars*) and GV$^{-/-}$ (*filled bars*) mice after NaCl or *E. coli* administration. *a*, $p < 0.05$, *E. coli versus* sham operation; *b*, $p < 0.05$, GV$^{+/+}$ *versus* GV$^{-/-}$, ANOVA followed by paired *t* test, 2-tailed, unequal variance, $n = 8$ per group. Immunohistochemical analysis with irrelevant primary isotype control antibody (*E* and *F*) or with specific rabbit primary antibodies against ICAM-1 (*G* and *I*) and PECAM-1 (*H* and *J*) in lungs 18 h after intra-tracheal injection of *E. coli* into GV$^{+/+}$ or GV$^{-/-}$ mice (×400) is shown. Magnified views (×1200) for ICAM-1 and PECAM-1 are shown in *panels I* and *J*, respectively. Representative images from five independent experiments are shown.

identified between GV$^{+/+}$ and GV$^{-/-}$ mice 18 h after *E. coli* infection (Fig. 6, *A–L*).

To independently evaluate eicosanoid levels in BAL fluid from GV$^{+/+}$ and GV$^{-/-}$ mice after sham operation or *E. coli* infection, we assessed PGD_2, PGE_2, and LTB_4 levels in BAL fluid by ELISA. This analysis confirmed that infection with *E. coli* stimulated an increase in the levels of PGD_2, PGE_{2v} and LTB_4 in the BAL fluid of both GV$^{+/+}$ and GV$^{-/-}$ mice in comparison with sham-operated animals. In addition, analysis by ELISA confirmed that there was no difference in the levels of PGD_2, PGE_2, or LTB_4, or the cysteinyl leukotrienes (LTC_4 and LTD_4) in BAL fluid after sham operation or after infection with *E. coli* between GV$^{+/+}$ and GV$^{-/-}$ mice (Table 2).

Analysis of eicosanoid levels in lung tissue by LC/MS/MS (after BAL fluid harvest, Fig. 7, *A–L*) demonstrated that levels of PGD_2 (Fig. 7*A*), $PGF_{2\alpha}$ (Fig. 7*D*), and 15-keto PGE_2 (Fig. 7*E*) were significantly higher in the lungs of GV$^{+/+}$ than GV$^{-/-}$ mice 18 h after *E. coli* infection. Levels of PGE_2 (Fig. 7*B*), LTB_4 (Fig. 7*I*), and 5-HETE (Fig. 7*J*) were significantly higher, and levels of 11-HETE (Fig. 7*K*) were significantly lower in lung tissue from GV$^{+/+}$ mice after *E. coli* infection than after sham operation, but there was no difference in the levels of these or

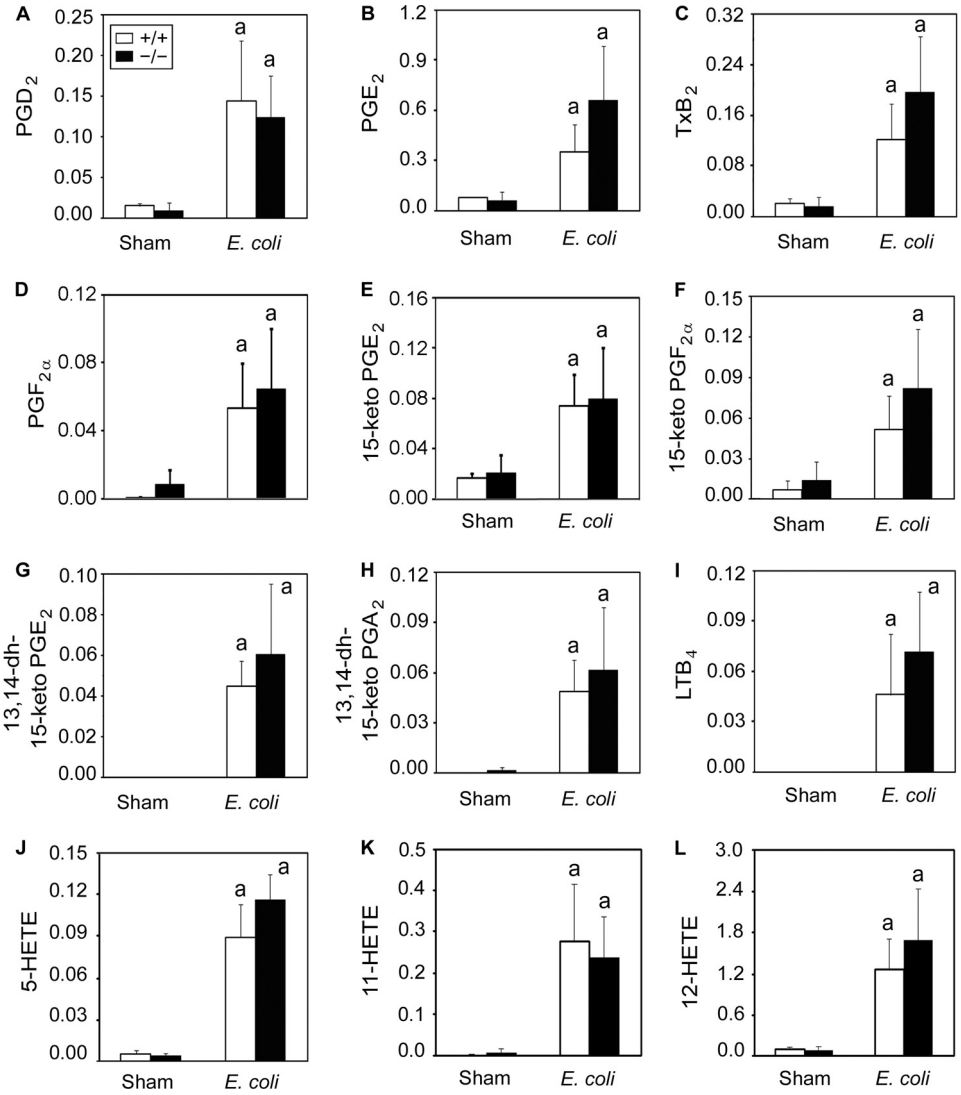

FIGURE 6. **Targeted deletion of GV sPLA$_2$ does not alter eicosanoid levels in BAL fluid after *E. coli* infection.** Eighteen hours after injection of 0.9% NaCl (sham operation) or 10^9 cfu of *E. coli* into the trachea of GV$^{+/+}$ (*open bars*) or GV$^{-/-}$ (*filled bars*) mice, eicosanoid levels in BAL fluid were analyzed by LC/MS/MS. PGD_2 (*A*), PGE_2 (*B*), (TxB_2 (*C*), $PGF_{2\alpha}$ (*D*), 15-keto PGE_2 (*E*), 15-keto $PGF_{2\alpha}$ (*F*), 13,14-dh-15-keto PGE_2 (*G*), 13,14-dh-15-keto PGA_2 (*H*), LTB_4 (*I*), 5-HETE (*J*), 11-HETE (*K*), and 12-HETE levels (*L*), expressed as ng/ml BAL fluid. *a*, $p < 0.05$, *E. coli versus* sham operation; ANOVA followed by paired *t* tests, 2-tailed, unequal variance, $n = 8 - 10$ independent experiments for each group.

TABLE 2

GV sPLA$_2$ does not regulate eicosanoid production in BAL fluid after infection with *E. coli*

GV$^{+/+}$ or GV$^{-/-}$ mice were subjected to intra-tracheal injection of NaCl or 10^9 CFU *E. coli*. After 18 h, BAL fluid was harvested, and the cysteinyl leukotrienes, PGD_2 methoxylamine hydrochloride (PGD_2-MOX), PGE_2 and PGE_2 metabolites, and LTB_4 were measured by ELISA. All values are expressed as pg/ml BAL fluid and are the result of ≥8 independent experiments for *E. coli* and ≥3 independent experiments for NaCl-injected mice.

Eicosanoid	GV$^{+/+}$ NaCl	GV$^{-/-}$ NaCl	GV$^{+/+}$ *E. coli*	GV$^{-/-}$ *E. coli*
Cysteinyl leukotrienes (LTC_4, LTD_4)	42 ± 10	46 ± 1	3,435 ± 889[a]	8,327 ± 1,977[a]
PGD_2-MOX	37 ± 12	44 ± 14	97.2 ± 9.7[a]	117.4 ± 14.0[a]
PGE_2	41 ± 18	62 ± 45	85.0 ± 13.6[a]	111 ± 27.1[a]
PGE_2 metabolite	58 ± 34	21 ± 5	135 ± 22.1[a]	92.9 ± 18.2[a]
LTB_4	59 ± 26	62 ± 33	153 ± 22.4[a]	119 ± 18.2[a]

[a] $p < 0.05$ *vs.* base line.

other eicosanoids (Fig. 7) in lung tissue from GV$^{+/+}$ and GV$^{-/-}$ mice 18 h after *E. coli* infection.

Lack of GV sPLA$_2$ in Non-myeloid Cells Impairs Leukocyte Accumulation after Pulmonary E. coli Infection—GV sPLA$_2$ is expressed by pulmonary epithelial cells and by inflammatory leukocytes after lung infection with *E. coli* (*cf.* Fig. 2). To elucidate the role of GV sPLA$_2$ in bone marrow-derived myeloid and non-myeloid cells in the regulation of leukocyte recruitment to lung and *E. coli* clearance after pulmonary infection, we generated chimeric female GV$^{+/+}$ and GV$^{-/-}$ mice with bone marrow from male GV$^{+/+}$ or GV$^{-/-}$ mice. After a 10-week interval to allow full humoral reconstitution, chimeras were infected with *E. coli*. The percentage of bone marrow cells from donor bone marrow, estimated by the ratio of SYR to GAPDH DNA in these cells, ranged from 89 to 99% and did not differ significantly between the four groups of chimeras (Fig. 8*A*). Similarly,

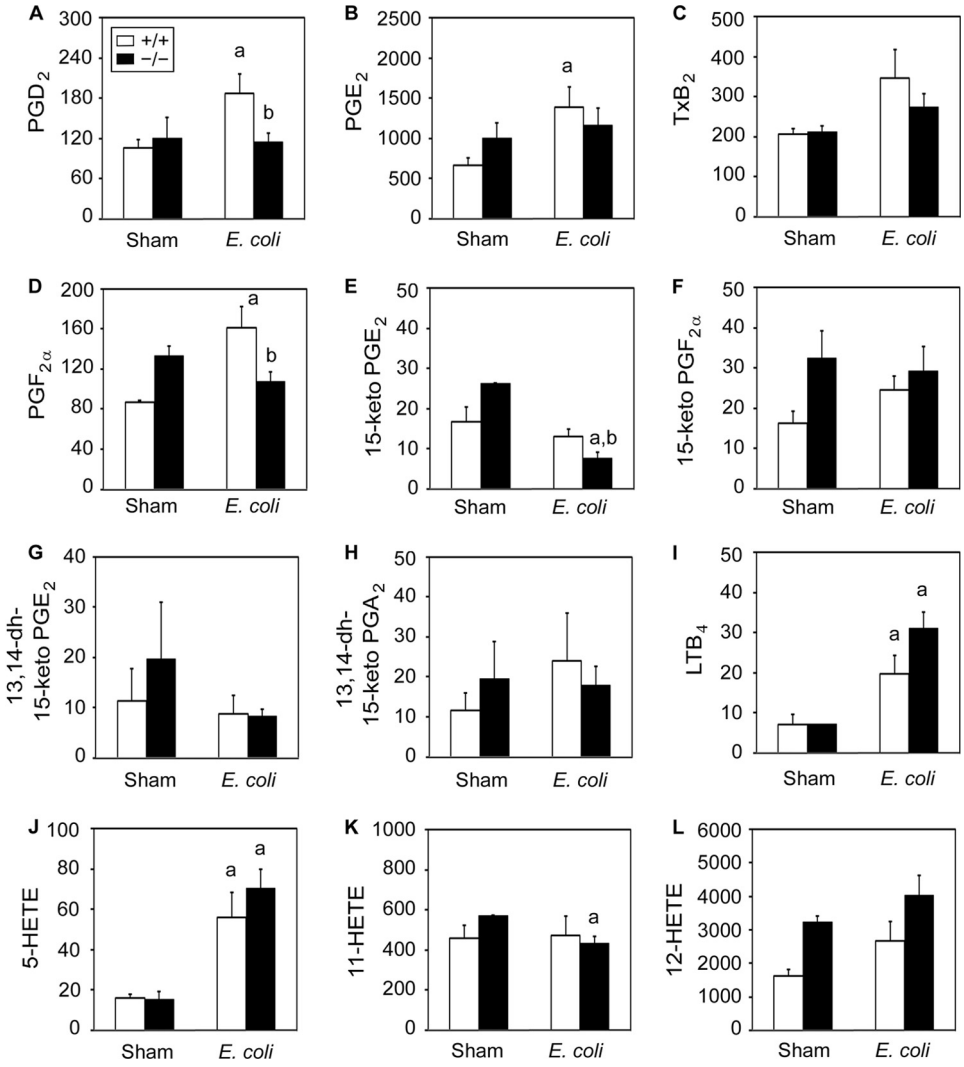

FIGURE 7. **Targeted deletion of GV sPLA$_2$ modulates the levels of selected eicosanoids in lung after *E. coli* infection.** Eighteen hours after injection of 0.9% NaCl (sham operation) or 10^9 cfu of *E. coli* into the trachea of GV$^{+/+}$ (*open bars*) or GV$^{-/-}$ (*filled bars*) mice, eicosanoid levels in lung tissue (after BAL fluid harvest) were analyzed by LC/MS/MS. PGD_2 (*A*), PGE_2 (*B*), TxB_2 (*C*), $PGF_{2\alpha}$ (*D*), 15-keto PGE_2 (*E*), 15-keto $PGF_{2\alpha}$ (*F*), 13,14-dh-15-keto PGE_2 (*G*), 13,14-dh-15-keto PGA_2 (*H*), LTB_4 (*I*), 5-HETE (*J*), 11-HETE (*K*), and 12-HETE levels (*L*), expressed as pg/mg of lung tissue. *a*, $p < 0.05$, *E. coli versus* sham operation or base line; *b*, $p < 0.05$, GV$^{+/+}$ *versus* GV$^{-/-}$, ANOVA followed by paired *t* tests, 2-tailed, unequal variance, $n = 6 - 8$ independent experiments for each group.

in these chimeras the percentage of alveolar macrophages from donor bone marrow ranged from 82 to 93% and also did not differ significantly between the 4 groups of chimeras (Fig. 8*B*). Therefore, in these chimeras cells in bone marrow and alveolar macrophages are derived predominantly from donor mice.

Levels of inflammatory cells in BAL fluid after *E. coli* infection tended to be higher in GV$^{+/+}$ mice with GV$^{+/+}$ bone marrow than in GV$^{+/+}$ mice with GV$^{-/-}$ bone marrow, but this difference was not statistically significant (Fig. 8*C*). In contrast, levels of inflammatory cells in BAL fluid were significantly higher in GV$^{+/+}$ mice with GV$^{+/+}$ bone marrow than in GV$^{-/-}$ mice with GV$^{+/+}$ bone marrow (Fig. 8*C*). Similarly, levels of inflammatory cells in BAL fluid were significantly higher in GV$^{+/+}$ mice with GV$^{-/-}$ bone marrow than in GV$^{-/-}$ mice with GV$^{-/-}$ bone marrow (Fig. 8*C*). Overall, levels of inflammatory cells in BAL fluid were significantly higher in GV$^{+/+}$ recipient mice (with either GV$^{+/+}$ or GV$^{-/-}$ bone marrow, $n = 13$) than in GV$^{-/-}$ recipient mice (with either GV$^{+/+}$ or GV$^{-/-}$ bone marrow, $n = 20$) after *E. coli* infection ($p = 0.021$, paired *t* test, two-tailed, unequal variance, Fig. 8*C*). Eighteen hours after lung infection with *E. coli*, the predominant inflammatory cell in BAL fluid from these chimeras was PMN, and there was no difference in the percentage of PMN in BAL fluid between any of the four chimeras (Fig. 8*D*). Taken together, these results provide direct evidence that GV sPLA$_2$ in non-myeloid pulmonary cells participates in the regulation of leukocyte accumulation in alveoli after lung infection with *E. coli*.

Lack of GV sPLA$_2$ in Bone Marrow-derived Myeloid Cells or Non-myeloid Cells Impairs Bacterial Clearance after Pulmonary E. coli Infection—Analysis of BAL fluid demonstrated similar levels of *E. coli* in GV$^{+/+}$ mice with GV$^{+/+}$ bone marrow and GV$^{+/+}$ mice with GV$^{-/-}$ bone marrow 18 h after pulmonary infection (Fig. 8*E*). In contrast, *E. coli* levels were significantly higher in BAL fluid from GV$^{-/-}$ mice with GV$^{+/+}$ bone marrow than GV$^{+/+}$ mice with GV$^{+/+}$ bone marrow and from GV$^{-/-}$ mice with GV$^{-/-}$ bone marrow than GV$^{+/+}$ mice with GV$^{-/-}$ bone marrow (after BAL fluid harvest, Fig. 8*E*). Overall,

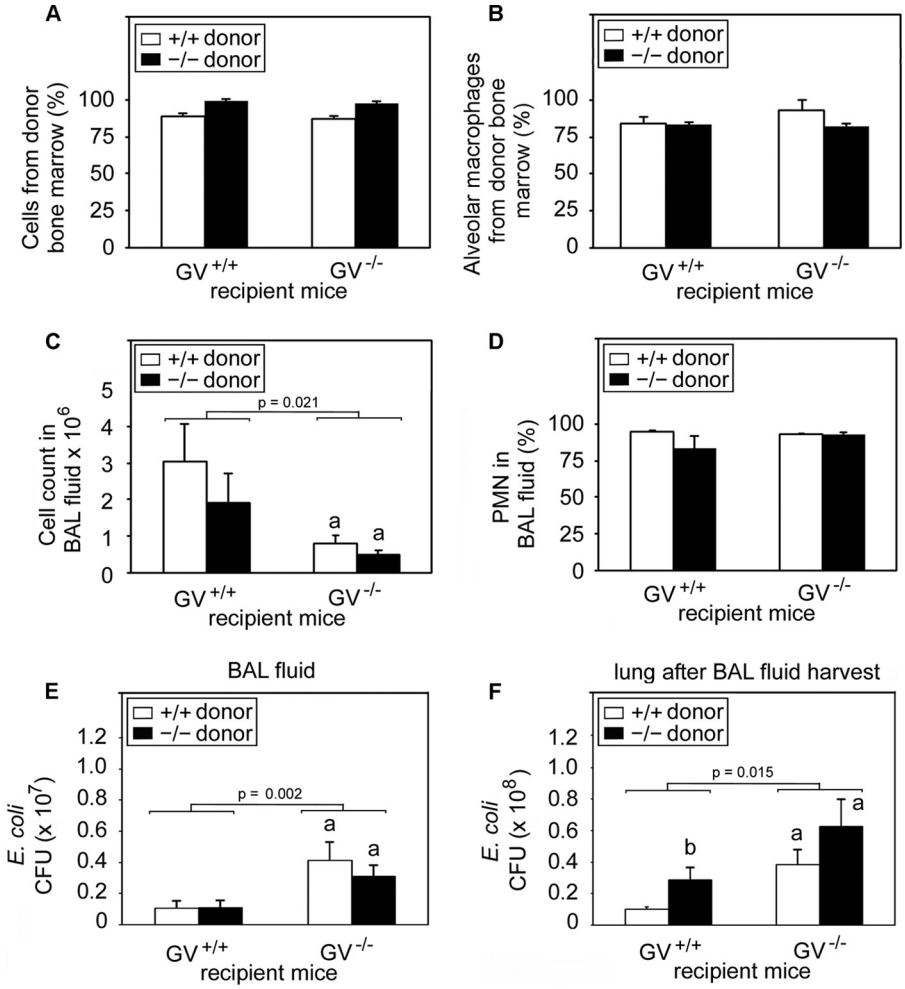

FIGURE 8. **Lack of GV sPLA$_2$ in non-myeloid cells attenuates alveolar leukocyte accumulation and lung *E. coli* clearance.** After irradiation, female GV$^{+/+}$ and GV$^{-/-}$ recipient mice were transplanted with bone marrow from male GV$^{+/+}$ (*open bars*) or GV$^{-/-}$ (*filled bars*) donor mice. The status of the recipient mice, GV$^{+/+}$ or GV$^{-/-}$, is indicated on the *x* axis of all panels in this figure. Shown is the percent of cells in bone marrow (*A*) and the percent of alveolar macrophages in GV$^{+/+}$ and GV$^{-/-}$ chimeras from donor bone marrow (*B*). Shown are cell counts (*C*), percentage of cells in BAL fluid that are PMN (*D*), bacterial viability (*colony forming units*) in BAL fluid (*E*), and lung after BAL fluid harvest (*F*) 18 h after intra-tracheal injection of 10^9 cfu of *E. coli*. *a*, $p < 0.05$, GV$^{+/+}$ mice with GV$^{+/+}$ bone marrow ($n = 7$) *versus* GV$^{-/-}$ mice with GV$^{+/+}$ bone marrow ($n = 10$) or GV$^{+/+}$ mice with GV$^{-/-}$ bone marrow ($n = 7$) *versus* GV$^{-/-}$ mice with GV$^{-/-}$ bone marrow ($n = 10$). *b*, $p < 0.05$, GV$^{+/+}$ mice with GV$^{+/+}$ bone marrow *versus* GV$^{+/+}$ mice with GV$^{-/-}$ bone marrow or GV$^{-/-}$ mice with GV$^{+/+}$ bone marrow *versus* GV$^{-/-}$ mice with GV$^{-/-}$ bone marrow; ANOVA followed by paired *t* test, 2-tailed, unequal variance.

bacterial levels in BAL fluid were significantly higher in GV$^{-/-}$ recipient chimeras (with either GV$^{+/+}$ or GV$^{-/-}$ bone marrow, $n = 20$) than in GV$^{+/+}$ recipient chimeras (with either GV$^{+/+}$ or GV$^{-/-}$ bone marrow, $n = 13$) 18 h after *E. coli* infection ($p = 0.002$, paired *t* test, two-tailed, unequal variance; Fig. 8*E*).

Analysis of lung tissue (after BAL fluid harvest) demonstrated significantly higher levels of *E. coli* in GV$^{+/+}$ mice with GV$^{-/-}$ bone marrow than GV$^{+/+}$ mice with GV$^{+/+}$ bone marrow 18 h after pulmonary infection (Fig. 8*F*). *E. coli* levels were also significantly higher in the lungs of GV$^{-/-}$ mice with GV$^{+/+}$ bone marrow than GV$^{+/+}$ mice with GV$^{+/+}$ bone marrow and the lungs of GV$^{-/-}$ mice with GV$^{-/-}$ bone marrow than GV$^{+/+}$ mice with GV$^{-/-}$ bone marrow (after BAL fluid harvest; Fig. 8*F*). Overall, bacterial levels in the lungs were higher in GV$^{-/-}$ recipient chimeras (with either GV$^{+/+}$ or GV$^{-/-}$ bone marrow, $n = 20$) than in GV$^{+/+}$ recipient chimeras (with either GV$^{+/+}$ or GV$^{-/-}$ bone marrow, $n = 13$) 18 h after *E. coli* infection ($p = 0.015$, paired *t* test, two-tailed, unequal variance, Fig. 8*F*). Taken together, these results provide direct evidence that GV sPLA$_2$ in non-myeloid cells regulates the clearance of *E. coli* from the alveolar space and that GV sPLA$_2$ in both bone marrow-derived myeloid cells and non-myeloid cells participates in the regulation of bacterial clearance from pulmonary parenchyma after lung infection with *E. coli*.

DISCUSSION

Multiple independent observations support a role for GV sPLA$_2$ in the immune response to infection. GV sPLA$_2$ is a component of primary and secondary granules in PMN and is released by PMN after exposure to the bacterial tripeptide, formylmethionylleucylphenylalanine (6). Peritoneal macrophages recruit GV sPLA$_2$ to the phagosome upon ingestion of zymosan (7), GV sPLA$_2$ regulates TLR2-stimulated eicosanoid biosynthesis by mast cells (31) and peritoneal macrophages (32), and macrophages from GV$^{-/-}$ mice exhibit impaired phagocytosis and killing of fungal particles (32). We sought to determine whether the role of GV sPLA$_2$ in innate immunity

extends to the clearance of a Gram-negative pathogen. Our data provide direct evidence that GV sPLA$_2$ is important for leukocyte recruitment to lung and for efficient pulmonary clearance of bacteria in a mouse model of *E. coli* pneumonia.

We noted that GV sPLA$_2$ is expressed by inflammatory cells recruited to the alveolar space and by bronchial epithelial cells (*cf.* Fig. 2) and that GV$^{-/-}$ mice clear bacteria from lung parenchyma and the alveolar space less efficiently than GV$^{+/+}$ mice 18 h after pulmonary infection with *E. coli*. A limitation of our initial studies with gene-targeted mice is that they did not allow us to determine if GV sPLA$_2$ in inflammatory cells or bronchial epithelial cells or both regulates leukocyte accumulation and bacterial clearance after lung infection with *E. coli*. To address this question, we generated chimeric mice that lack GV sPLA$_2$ in myeloid and/or non-myeloid cells and evaluated the response of these mice to lung *E. coli* infection. The studies that document significantly higher levels of *E. coli* in the lung parenchyma of GV$^{+/+}$ mice with GV$^{-/-}$ bone marrow than GV$^{+/+}$ mice with GV$^{+/+}$ bone marrow constitute the first demonstration that GV sPLA$_2$ in bone marrow-derived myeloid cells directly regulates the clearance of a pathogen, in this case *E. coli*, *in vivo*.

Our data also identify multiple independent lines of evidence that support a role for GV sPLA$_2$ in non-myeloid cells in pulmonary *E. coli* clearance. Levels of *E. coli* were significantly higher in lung tissue (after BAL fluid harvest) of chimeric GV$^{-/-}$ mice with GV$^{+/+}$ bone marrow than in GV$^{+/+}$ mice with GV$^{+/+}$ bone marrow and were also significantly higher in the lungs of chimeric GV$^{-/-}$ mice with GV$^{-/-}$ bone marrow than GV$^{+/+}$ mice with GV$^{-/-}$ bone marrow. Overall, *E. coli* levels were significantly higher in BAL fluid from GV$^{-/-}$ recipient chimeras (with either GV$^{+/+}$ or GV$^{-/-}$ bone marrow) than GV$^{+/+}$ recipient chimeras (with either GV$^{+/+}$ or GV$^{-/-}$ bone marrow). Together, these results provide direct evidence that GV sPLA$_2$ in non-myeloid cells directly regulates bacterial clearance after lung infection with *E. coli*. GV sPLA$_2$ can be expressed by multiple cells in the lung, including bronchial epithelial cells (*cf.* Fig. 2), airway smooth muscle cells (33), lung fibroblasts (13), PMN, and alveolar macrophages (Fig. 2 and supplemental Fig. 3). As bronchial epithelial cells are the only non-myeloid cells in the lungs that appear to express GV sPLA$_2$ in these mice, our results are consistent with the notion that GV sPLA$_2$ in bronchial epithelial cells regulates bacterial clearance after lung infection with *E. coli*.

GV sPLA$_2$ can hydrolyze *E. coli* phospholipids *in vitro* (6, 28). We noted that PLA$_2$ activity is higher in BAL fluid from GV$^{+/+}$ than GV$^{-/-}$ mice after *E. coli* infection and that clearance of bacteria from the lung is attenuated in GV$^{-/-}$ in comparison with GV$^{+/+}$ mice. Therefore, it is possible that GV sPLA$_2$ released by myeloid or bronchial epithelial cells may be necessary to efficiently kill *E. coli in vivo*. However, increased GV sPLA$_2$ levels in BAL fluid may not be sufficient to kill *E. coli* because direct exposure of live *E. coli* to recombinant GV sPLA$_2$ had no effect on bacterial viability *in vitro*. As endogenous GV sPLA$_2$ released from stimulated epithelial cells can translocate to eosinophils and promote eosinophil adhesion to ICAM-1 and eicosanoid biosynthesis (34), it is possible that GV sPLA$_2$ released by myeloid cells or bronchial epithelial cells regulates bacterial clearance through paracrine regulation of inflammatory cells in the interstitial microenvironment.

Lower PMN accumulation in the alveolar space of GV$^{-/-}$ than GV$^{+/+}$ mice after lung infection with *E. coli* (*cf.* Fig. 4*D*) may also explain why bacterial clearance was impaired in GV$^{-/-}$ mice, because PMN efficiently ingest and kill *E. coli in vitro* (35). Attenuated accumulation of inflammatory cells in the alveolar space of GV$^{-/-}$ in comparison with GV$^{+/+}$ mice may be due to decreased expression of ICAM-1 and/or PECAM-1 on pulmonary endothelial cells in GV$^{-/-}$ mice (*cf.* Fig. 5), as these adhesion molecules regulate leukocyte transmigration from blood to the alveolar space (36–38). In support of this notion, inhibition of sPLA$_2$ activity with 12-epi-scalaradial attenuates TNF-α mediated ICAM-1 expression by HaCaT keratinocytes (39), and targeted deletion of GV SPLA$_2$ decreases ICAM-1 and VCAM-1 expression in a murine air pouch model after exposure to LPS (40). It is possible that expression of ICAM-1 and PECAM-1 and migration of PMN from blood to the alveolar space are partially regulated by eicosanoid synthesis linked to GV sPLA$_2$ in bronchial epithelial cells because PGD$_2$, PGF$_{2\alpha}$, and 15-keto-PGE$_2$ levels in lung parenchyma are lower in GV$^{-/-}$ than GV$^{+/+}$ mice 18 h after lung infection with *E. coli*, and these eicosanoids have been implicated in leukocyte recruitment to sites of inflammation *in vivo* (41, 42).

As sPLA$_2$ enzymes function upstream from COX and terminal eicosanoid synthase enzymes (15), it is not clear how targeted deletion of GV sPLA$_2$ led to a reduction in the levels of selected eicosanoids in lung parenchyma after infection with *E. coli*. Another limitation of this study is that we are not able to determine if alterations in a single or multiple eicosanoids or alterations in other factors not related to eicosanoid biosynthesis decrease ICAM-1 and PECAM-1 expression in the lungs of GV$^{-/-}$ in comparison with GV$^{+/+}$ mice after *E. coli* infection. These limitations notwithstanding, the independent observations that the levels of inflammatory cells in BAL fluid are higher in GV$^{+/+}$ mice with GV$^{+/+}$ bone marrow than in GV$^{-/-}$ mice with GV$^{+/+}$ bone marrow and are also higher in BAL fluid in GV$^{+/+}$ mice with GV$^{-/-}$ bone marrow than in GV$^{-/-}$ mice with GV$^{-/-}$ bone marrow constitute the first demonstration that GV sPLA$_2$ in non-myeloid cells, likely bronchial epithelial cells, directly regulates alveolar leukocyte accumulation after lung infection with *E. coli*. The observation that leukocyte levels in the alveolar space are lower in GV$^{-/-}$ than GV$^{+/+}$ mice after intra-tracheal administration of LPS (43)[6], sensitization, and challenge with ovalbumin (11), and *E. coli* infection (*cf.* Fig. 4*D*) suggests a general role for GV sPLA$_2$ in bronchial epithelial cells in pulmonary leukocyte recruitment.

There are other mechanisms that could explain decreased accumulation of PMN in the alveolar space of GV$^{-/-}$ mice after *E. coli* infection. For example, increased human pulmonary endothelial cell permeability induced by LPS *in vitro* is partially mediated by GV sPLA$_2$ (44), so it is possible that a lack of GV sPLA$_2$ preserves endothelial cell barrier integrity and impedes leukocyte transmigration into the alveolar space in GV$^{-/-}$ in comparison with GV$^{+/+}$ mice after lung infection with *E. coli*.

[6] N. Degousee, D. J. Kelvin, and B. B. Rubin, unpublished observations.

Eicosanoids such as LTB_4, TxA_2, and PGE_2 promote leukocyte recruitment to lung (45). Although significantly less LTB_4 and PGE_2 is produced by macrophages from $GV^{-/-}$ than $GV^{+/+}$ mice (16) and $GV^{-/-}$ mice have lower levels of LTE_4 in the peritoneal cavity after injection of zymosan than $GV^{+/+}$ mice (16), we failed to identify differences in the levels of any eicosanoids in BAL fluid, measured independently by LC/MS/MS and ELISA, between $GV^{+/+}$ and $GV^{-/-}$ mice after lung infection with *E. coli*. PMN migration into the alveolar space after pulmonary *E. coli* infection is dependent on CD11b/CD18 on the surface of PMN (30). Although exogenous human GV $sPLA_2$ can increase surface expression of CD11b and focal clustering of this integrin on eosinophils (34), we also failed to identify differences in CD11b/CD18 expression on the surface of PMN in the alveolar space of $GV^{+/+}$ and $GV^{-/-}$ mice after lung *E. coli* infection. Decreased levels of PMN in the alveolar space of $GV^{-/-}$ *versus* $GV^{+/+}$ mice is not due to differences in PMN apoptosis between these mice after migration of PMN into alveoli or to a lack of GV $sPLA_2$ in these cells, because levels of PMN in BAL fluid were similar in $GV^{+/+}$ mice with $GV^{+/+}$ bone marrow and $GV^{+/+}$ mice with $GV^{-/-}$ bone marrow after lung infection with *E. coli*. Therefore, differences in eicosanoid levels in BAL fluid, expression of CD11b/CD18 on the surface of PMN, or PMN apoptosis between $GV^{+/+}$ and $GV^{-/-}$ mice do not explain the difference in PMN accumulation and *E. coli* clearance from BAL fluid observed between $GV^{+/+}$ and $GV^{-/-}$ mice after pulmonary *E. coli* infection.

The defect in pulmonary *E. coli* clearance in $GV^{-/-}$ mice is physiologically important, because the 3-fold increase in *E. coli* levels in the lung parenchyma of $GV^{-/-}$ *versus* $GV^{+/+}$ mice is sufficient to drive a systemic inflammatory response, manifest by more profound hypothermia, a characteristic response of mice to bacterial infection (29), relative leukocytosis, and higher levels of IL-6, IL-10, and TNF-α in arterial blood from mice lacking GV $sPLA_2$. Our finding are consistent with the observation that IL-6 and TNF-α promote post-infection hypothermia in mice (46, 47) but are at odds with the findings that IL-10 attenuates the hypothermic response to infection and inhibits LPS-induced production of TNF-α and IL-6 (48) and that IL-$10^{-/-}$ mice exhibit more profound hypothermia than IL-$10^{+/+}$ mice after LPS injection (49), Although our studies have not identified the molecular mechanisms responsible for the more profound hypothermia and systemic inflammation observed in $GV^{-/-}$ mice after lung infection with *E. coli*, they do show that an isolated increase in *E. coli* in lung parenchyma is sufficient to drive a systemic inflammatory response in these mice, because levels of *E. coli* in other organs, including liver, spleen, and kidneys as well as arterial blood are similar in $GV^{+/+}$ and $GV^{-/-}$ mice after pulmonary *E. coli* infection.

In addition to participating in the innate immune response to Gram-negative bacterial and fungal (32) infections, GV $sPLA_2$ plays a role in the development and progression of atherosclerosis and has been identified in both human and mouse atherosclerotic lesions (50). Human low density lipoprotein modified by GV $sPLA_2$ is pro-atherogenic and promotes macrophage foam cell formation and overexpression of GV $sPLA_2$ increases, and targeted deletion of GV $sPLA_2$ decreases atherosclerotic plaque size in low density lipoprotein receptor-deficient mice (51, 52). Based on these observations, pharmacologic inhibition of GV $sPLA_2$ has been identified as a possible therapeutic strategy for patients with atherosclerosis (21, 53). Our observations suggest that the benefits of pharmacologic inhibition of GV $sPLA_2$ in the treatment of atherosclerosis and other inflammatory diseases should be balanced against the potential risk of attenuating the innate immune response to pulmonary Gram-negative bacterial and fungal (32) infections in patients exposed to such pharmacologic therapies.

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